**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 2/00, 14/705, 16/28, C12N 5/06, 5/07, C12Q 1/00, A16K 31/00, 35/00, 39/395, G01N 33/15, 33/48, 22/53

A1

(11) International Publication Number: WO 97/34920

(43) International Publication Date: 25 September 1997 (25.09.97)

(21) International Application Number: PCT/US97/03378

(22) International Filing Date: 4 March 1997 (04.03.97)

(30) Priority Data:
08/621,734 21 March 1996 (21.03.96) US

(71) Applicant: SUGEN, INC. [US/US]; 515 Galveston Drive, Redwood City, CA 94063 (US).

(72) Inventors: HIRTH, Klaus, P.; 334 Collingwood, San Francisco, CA 94114 (US). MCMAHON, Gerald; 1414 Greenwich Street, San Francisco, CA 94109 (US). SHAWVER, Laura, K.; 216 Cotter Street, San Francisco, CA 94112 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds L.L.P., 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GH, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: ASSAYS FOR KDR/FLK-1 RECEPTOR TYROSINE KINASE INHIBITORS

(57) Abstract

The present invention relates to processes for the identification of compounds and pharmaceutical compositions capable of selectively and potently inhibiting KDR/FLK-1 tyrosine kinase signal tranduction in order to inhibit vasculogenesis and/or angiogenesis. The present invention further relates to compounds and compositions identified using the methods of the invention and the use thereof for the treatment of disease relating to inappropriate vasculogenesis and/or angiogenesis.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

"ASSAYS FOR KDR/FLK-1 RECEPTOR TYROSINE KINASE INHIBITORS"

## 1. FIELD OF THE INVENTION

The present invention relates to methods for the identification and use of compounds capable of selectively and potently inhibiting the signal transduction of a specific receptor tyrosine kinase, and more particularly to compounds selectively inhibiting the enzymatic function of a VEGF receptor, *e.g.*, the KDR/FLK-1 receptor, and the compounds so identified.

## 2. BACKGROUND OF THE INVENTION

***Receptor Tyrosine Kinases.*** Receptor tyrosine kinases (RTKs) comprise a large family of transmembrane receptors for polypeptide growth factors with diverse biological activities. The intrinsic function of RTKs is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses. Ullrich and Schlessinger, 1990, *Cell 61*:203-212.

As has been reported, RTKs, as well as, more generally, protein tyrosine kinases, play an important role in the control of cell growth and differentiation (for review, *see*, Schlessinger and Ullrich, 1992, *Neuron 9*:383-391). This is reflected in the observation that aberrant expression or mutations in members of the RTK family lead to either uncontrolled cell proliferation (*e.g.*, malignant tumor growth) or to defects in key developmental processes. Consequently, the biomedical community has expended significant resources to discover the specific

biological role of members of the RTK family, their function in differentiation processes, including their involvement in tumorigenesis and in other diseases, the biochemical mechanisms underlying their signal transduction pathways activated upon ligand stimulation and the development of novel antineoplastic drugs.

At present, at least nineteen (19) distinct RTK subfamilies have been identified. One RTK subfamily is believed to be comprised of the KDR/FLK-1 receptor, the fetal liver kinase 4 (FLK-4) receptor and the fms-like tyrosine 1 (flt-1) receptor. Each of these receptors was initially believed to be receptors for hematopoietic growth factors.

***The KDR/FLK-1 Receptor and VEGF.*** Normal vasculogenesis and angiogenesis play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Folkman and Shing, 1992, *J. Biological Chem.* *267*:10931-34. However, many diseases are driven by persistent unregulated or inappropriate angiogenesis. For example, in arthritis, new capillary blood vessels invade the joint and destroy the cartilage. In diabetes, new capillaries in the retina invade the vitreous, bleed and cause blindness. Folkman, 1987, in: *Congress of Thrombosis and Haemostasis* (Verstraete, *et. al*, eds.), Leuven University Press, Leuven, pp.583-596. Ocular neovascularization is the most common cause of blindness and dominates approximately twenty (20) eye diseases.

Moreover, vasculogenesis and/or angiogenesis have been associated with the growth of malignant solid tumors and metastasis. A tumor must continuously

stimulate the growth of new capillary blood vessels for the tumor itself to grow. Furthermore, the new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation and to metastasize to distant sites in the body. Folkman, 1990, *J. Natl. Cancer Inst. 82*:4-6; Klagsbrunn and Soker, 1993, *Current Biology 3*:699-702; Folkman, 1991, *J. Natl., Cancer Inst. 82*:4-6; Weidner *et al.*, 1991, *New Engl. J. Med. 324*:1-5.

Several polypeptides with *in vitro* endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor (aFGF, bFGF), vascular endothelial growth factor (VEGF) and placental growth factor. Unlike aFGF and bFGF, VEGF has recently been reported to be an endothelial cell specific mitogen. Ferrara and Henzel, 1989, *Biochem. Biophys. Res. Comm. 161*:851-858; Vaisman *et al.*, 1990, *J. Biol. Chem. 265*:19461-19566.

Thus, the identification of the specific receptors to which VEGF binds is an important advancement in the understanding of the regulation of endothelial cell proliferation. Two structurally closely related RTKs have been identified to bind VEGF with high affinity: the flt-1 receptor (Shibuya *et al.*, 1990, *Oncogene 5*:519-524; De Vries *et al.*, 1992, *Science 255*:989-991) and the KDR/FLK-1 receptor, discussed in the U.S. Patent Application No. 08/193,829. Consequently, it had been surmised that these RTKs may have a role in the modulation and regulation of endothelial cell proliferation.

Evidence, such as the disclosure set forth in copending U.S. Application Serial No. 08/193,829, strongly suggests that VEGF is not only responsible for endothelial cell proliferation, but also is a prime regulator of normal

and pathological angiogenesis. *See generally*, Klagsburn and Soker, 1993, *Current Biology* 3:699-702; Houck *et al.*, 1992, *J. Biol. Chem.* 267:26031-26037. Moreover, it has been shown that KDR/FLK-1 and flt-1 are abundantly expressed in the proliferating endothelial cells of a growing tumor, but not in the surrounding quiescent endothelial cells. Plate *et al.*, 1992, *Nature* 359:845-848; Shweiki *et al.*, 1992, *Nature* 359:843-845.

***Identification Of Agonists And Antagonists To The KDR/FLK-1 Receptor.*** In view of the deduced importance of RTKs in the control, regulation and modulation of endothelial cell proliferation and potentially vasculogenesis and/or angiogenesis, many attempts have been made to identify RTK "inhibitors" using a variety of approaches. These include the use of mutant ligands (U.S. Patent No. 4,966,849); soluble receptors and antibodies (Application No. WO 94/10202; Kendall and Thomas, 1994, *Proc. Natl. Acad. Sci. USA* 90:10705-10709; Kim *et al.*, 1993, *Nature* 362:841-844); and RNA ligands (Jellinek *et al.*, 1994, *Biochemistry* 33:10450-10456). However, so far, none of these efforts have resulted in the identification or isolation of inhibitors useful for therapeutic applications.

Furthermore, tyrosine kinase inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Patent No. 5,330,992; Mariani *et al.*, 1994, *Proc. Am. Assoc. Cancer Res.* 35:2268), and inhibitors acting on receptor tyrosine kinase signal transduction pathways, such as protein kinase C inhibitors have been identified (Schuchter *et al.*, 1991, *Cancer Res.* 51:682-687); Takano *et al.*, 1993, *Mol. Bio. Cell* 4:358A; Kinsella *et al.*, 1992, *Exp. Cell Res.* 199:56-62; Wright *et al.*, 1992, *J. Cellular Phys.* 152:448-57).

More recently, attempts have been made to identify small molecules which act as tyrosine kinase inhibitors. For example, bis monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO 94/14808) and 1-cycloproppyl-4-pyridyl-quinolones (U.S. Patent No. 5,330,992) have been described generally as tyrosine kinase inhibitors. Styryl compounds (U.S. Patent No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660) and benzylphosphonic acid compounds (PCT WO 91/15495) have been described as compounds for use as tyrosine kinase inhibitors for use in the treatment of cancer. None of these compounds, however, have been demonstrated to selectively act on the enzymatic function of a specific receptor tyrosine kinase, such as the KDR/FLK-1 receptor. Indeed, because in a substantial percentage of tumors, overexpression of various RTKs had been shown to dictate the uncontrolled proliferation of the tumor cells these compounds have been identified with the objective to inhibit receptor tyrosine kinase function in general. Likewise, these compounds have not been associated with the selective inhibition of specific receptor tyrosine kinases, or the inhibition of vasculogenesis and/or angiogenesis.

Consequently, there is an unmet need for the identification and generation of effective small compounds which selectively inhibit the signal transduction of the KDR/FLK-1 receptor in order to effectively and specifically suppress vasculogenesis.

## 3. SUMMARY OF THE INVENTION

The present invention, for the first time, provides for a process to systematically and rationally identify compounds targeting specific biomolecules in order to affect a particular physiological process. More particularly, the present invention relates to a process for producing a compound that inhibits vasculogenesis and/or angiogenesis comprising screening a plurality of test compounds to identify a subset of compounds which selectively and potently inhibits the VEGF receptor.

The present invention relates to a process for the identification of compounds that are both highly potent and highly selective for inhibiting the activity of a VEGF receptor, *e.g.*, KDR/FLK-1, in order to inhibit vasculogenesis and/or angiogenesis. More specifically, the invention relates to an assay cascade comprised of several "filter steps" of increasing selectivity, which identify a limited subset of candidate compounds affecting the VEGF receptor on the molecular level. The assays comprising these filter steps are preferrably high-throughput type assays.

A "high-throughput" cellular assay is employed as the first filter step to identify subset candidate compounds inhibiting VEGF-induced tyrosine phosphorylation of KDR/FLK-1 in cultured cells. Controls are designed to determine the candidate compounds' selectivity for inhibition of KDR/FLK-1 relative to other receptor tyrosine kinases, for example, the IGF-1-R or the EGF-R. Subset candidate compounds with a $IC_{50}$ of <50 $\mu$M, and more preferably <10 $\mu$M, and with a selectivity for KDR/FLK-1 of twofold, and more preferably fivefold, relative to control receptors are further pursued. All other

"negative" compounds which do not meet these criteria are discarded.

In the second filter step a bioresponse assay is employed to determine the efficacy of the KDR/FLK-1 inhibitors in cultured endothelial cells. In one embodiment of the method of the invention, the human umbilical vein endothelial cell line HUV-EC-C is employed. HUV-EC cells express the KDR/FLK-1 receptor, and upon VEGF stimulation, a mitotic response can be measured. Controls are designed to determine the selectivity of inhibition of the compounds against the VEGF-induced bioresponse relative to the bioresponse mediated by other receptor tyrosine kinases expressed in the cultured endothelial cells, for example the receptor for aFGF may be compared. Subset candidate compounds with a efficacy of <50 $\mu$M, preferably <10 $\mu$M, and with a selectivity of inhibition twofold, preferably threefold relative to a control receptor, are determined to be "positive". Compounds identified as "positive" are saved for further testing; the "negative" compounds are removed.

Optionally, a biochemical assay may be employed as a "filter" step in order to test the subset candidate compounds' effect on the VEGF-induced autophosphorylation of KDR/FLK-1 in a cell free system. More specifically, the compounds identified as "positive" in the above cellular assay may be tested in a defined *in vitro* assay to confirm that the inhibitory effects are due to direct interaction of the subset candidate compounds with KDR/FLK-1 rather than the result of any secondary effects in the cell. The subset candidate compounds identified as "positive" are again saved for the next "filter."

All of the candidate compounds which survive as "positives" through the above described filter steps

are then tested for toxicity by determination of the $LD_{10}$ value and further for efficacy an *in vivo* model for angiogenesis. Suitable *in vivo* models are those for metastasis and or diabetic retinopathy. The exemplary model described herein is a subcutaneous tumor xenograph model. The compounds of the invention are those which are determined to inhibit tumor angiogenesis in this model, indicated by inhibition of the tumor growth by at least 30% relative to untreated controls, at a dose smaller than the $LD_{10}$.

Compounds of this invention include, for example, derivatives of quinazoline, quinoxiline, substituted aniline, indoline, isoxazoles, acrylonitrile and phenyl-acrylonitrile compounds. The compounds of the present invention, however, are not limited to certain chemical classes, as they are defined by the process of identification and their so determined physiological activity.

The present invention is further directed to pharmaceutical compositions comprising a pharmaceutically effective amount of the compounds of the invention and a pharmaceutically acceptable carrier or excipient. Such compositions are believed to specifically inhibit the KDR/FLK-1 receptor by, *e.g.*, inhibiting its catalytic activity, affinity to ATP or ability to interact with a substrate, and thus will be useful in inhibition of diseases related to vasculogenesis and/or angiogenesis, including diabetes, arthritis, and cancer.

Finally, the present invention is also directed to methods for treating diseases related to pathological or inappropriate vasculogenesis and/or angiogenesis, including but not limited to diabetes, diabetic retinopathy, rheumatoid arthritis, hemangioma and cancer and more particularly cancer related to solid cell tumor growth (*e.g.*, glioblastoma, melanoma

and Kaposi's sarcoma and ovarian, lung, mammary, prostate, pancreatic, colon and epidermoid carcinoma).

## 4. DEFINITIONS

The following terms, in singular and plural, are be intended to have, for the purpose of this invention, the following meaning.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases and which are obtained by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid and the like.

"Selectivity for", "selective inhibition of", or "selectively inhibit(ing)" an VEGF receptor, for example the KDR/FLK-1 receptor refers to the preferential inhibitory ability of a compound against a VEGF receptor compared to a receptor that belong to another RTK subfamily, such as for example the EGF-R.

"VEGF recptor" refers to a transmembrane protein with tyrosine kinase activity that binds VEGF, including KDR/FLK-1 and flt-1.

## 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to processes for the identification of compounds capable of selectively and potently inhibiting the activity of a VEGF receptor, *e.g.* KDR/FLK-1, useful in the treatment of diseases related to unregulated or inappropriate angiogenesis and/or vasculogenesis and the compounds so produced. More particularly, the present invention is based, in part, on the recent discovery that the KDR/FLK-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and may

play a role in endothelial cell growth, which is one of the crucial steps involved in vasculogenesis and/or angiogenesis. *See*, copending U.S. Patent Application No. 08/193,829, filed February 9, 1994, incorporated by reference in its entirety herein. The invention is also based on the identification of VEGF as a high affinity ligand of KDR/FLK-1 and the characterization of KDR/FLK-1 as a regulator of vasculogenesis and/or angiogenesis rather than a hematopoietic receptor. Thus, the surmised role of VEGF in endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicates an important role for the KDR/FLK-1 in these processes.

The invention is further based on the observation that an array of severe diseases, such as diabetic retinopathy (Folkman, 1987, in XIth Congress of Thrombosis and Haemostasis (Verstraeta *et al.*, eds.) pp. 583-596, Leuven University Press, Leuven) and arthritis, as well as malignant tumor growth involve, as an essential requirement for the disease to develop, uncontrolled or inappropriate angiogenesis. *See e.g.*, Folkman, 1971, *N. Engl. J. Med. 285*:1182-1186. For example, in arthritis, new capillaries invade the joint and destroy the cartilage; in at least twenty (20) ocular diseases, including diabetic retinopathy, new and bleeding capillaries cause blindness.

Moreover, a tumor must continuously stimulate the growth of new blood vessels for the tumor itself to grow. Also metastasis of malignant tumors has been directly associated with vasculogenesis and/or angiogenesis, as new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation in order to manifest at distant sites in the body.

While not wishing to be bound by any particular theory, although it is believed that the compounds of the present invention act on the endothelial cells forming new blood vessels during vasculogensis and/or angiogenesis, the compounds may also act directly on the tumors cells.

For purposes of this application, although the nomenclature of the human and murine counterparts of the generic "FLK-1" receptor differ slightly, they are, in many respects, interchangeable. Models which rely upon the FLK-1 receptor therefore are directly applicable to understanding the KDR receptor. The murine receptor, FLK-1, and its human counterpart, KDR, share a sequence homology of 93.4% within the intracellular domain which is responsible for the enzymatic activity and resulting signal transduction. Likewise, murine FLK-1 binds human VEGF with the same affinity as mouse VEGF, and accordingly, is activated by the ligand derived from either species. Millauer *et al.*, 1993, *Cell* 72:835-846; Quinn *et al.*, 1993, *Proc. Natl. Acad. Sci. USA* 90:7533-7537. FLK-1 also associates with and subsequently tyrosine phosphorylates human RTK substrates (*e.g.*, PLC-γ or p85) when coexpressed in 293 cells (human embryonal kidney fibroblasts).

Use of the murine FLK-1 receptor in methods to identify compounds which regulate the signal transduction pathway are directly applicable to the identification of compounds which may be used to regulate the human signal transduction pathway, and more specifically, activity related to the KDR receptor. Angiogenesis is a very complex process involving the invasion of endothelial cells into the nonvascularized tissue. No *in vitro* model exists which mimics exactly this multistep process comprising the degradation of the basal membrane surrounding the

endothelial cells, migration into the perivascular stroma and eventually proliferation and formation of the new vascular sprout. However, *in vivo* mouse and rat animal models have been demonstrated to be of excellent value for the examination of the clinical potential of agents acting on the KDR/FLK-1 induced signal transduction pathway.

In sum, the receptors to which VEGF specifically binds are an important and powerful therapeutical target for the regulation and modulation of vasculogenesis and/or angiogenesis and a variety of severe diseases which involve abnormal cellular growth caused by such processes. Plowman *et al.*, 1994, *DN&P* 7:334-339. More particularly, the KDR/FLK-1 receptor's high specificity and role in the neovascularization make it a very distinct and powerful target for therapeutic approaches for the treatment of cancer and other diseases which involve the uncontrolled or inappropriate formation of blood vessels.

This invention is therefore directed to the a process designed to systematically and rationally identify compounds which inhibit vasculogenesis and/or angiogenesis by selectively targeting and inhibiting VEGF receptor, *e.g.*, KDR/FLK-1, on a molecular level. The compounds produced by the process of the invention may inhibit the KDR/FLK-1 receptor by, *e.g.*, inhibiting its catalytic activity, affinity to ATP or ability to interact with a substrate.

### 5.1. The Assay Cascade For The Identification Of Compounds Selectively Inhibiting The KDR/FLK-1 Receptor Signal Transduction

The present invention, for the first time, provides for a process to systematically and rationally identify compounds targeting a VEGF receptor, *e.g.*, KDR/FLK-1, in order to affect its

physiological functionality, *i.e.*, vasculogenesis and/or angiogenesis. More specifically, the process of the invention allows one to produce, by way of identification, compounds selectively targeting KDR/FLK-1 for the treatment of diseases related to uncontrolled or inappropriate vasculogenis and/or angiogenesis.

More specifically, the invention relates to a cascade of assays including filter steps of increasing stringency for the identification of compounds which selectively and potently inhibit KDR/FLK-1 tyrosine kinase signal transduction. Using the process of this invention allows one to produce highly potent inhibitors which are highly specific for KDR/FLK-1 as molecular target. Acting on a specifically defined molecular target involved in the regulation of vasculogenesis and/or angiogenesis, the compounds and their pharmaceutical compositions containing them should have superior therapeutic value while having fewer undefined side effects than drugs identified by traditional means and screening methods.

The steps involved in cascades of assays are designed as "filter steps" of increasing stringency. For example, the first filter steps are cellular and/or biochemical high-throughput screening assays, respectively, allowing the identification of subset candidate compounds selectively targeting and potently inhibiting KDR/FLK-1 on the molecular level. The next filter step tests the subset candidate compounds' efficacy and selectivity of inhibition against a VEGF-induced bioresponse in cultured endothelial cells. Finally, a last filter step, the compounds are tested in an *in vivo* model of angiogenesis to select compounds that are both efficacious and relatively non-toxic as well as having an acceptable pharmacological profile. Models for diabetic

retinopathy or metastasis, both of which are well known in the art, would be appropriate. The present disclosure describes the use of a mouse subcutaneous xenograft model designed to identify compounds inhibiting tumor vasculogenesis and/or angiogenesis.

### 5.1.1. High Throughput Cellular Assay For The Identification Of KDR/FLK-1 Inhibitors

A first "filter step" of the assay cascade, is designed to allow for high-throughput testing of compounds from any source to identify molecules having an inhibitory effect on KDR/FLK-1 autophosphorylation.

For example, a cellular assays may be developed to screen for subset candidate compounds which inhibit KDR/FLK-1 autophosphorylation, for example using cultured cells expressing high levels of KDR/FLK-1 cDNA.

In a specific embodiment of the invention, NIH 3T3/FLK-1 cells are seeded in 96 well cell culture plates, grown to suitable density and then incubated with a test candidate compound. Subsequently, the cells are stimulated with VEGF as previously described (*see*, copending U.S. Patent Application 08/485,323, filed June 7, 1995), and lysed. The level of tyrosine phosphorylation is determined via Enzyme Linked Immunoabsorbent Assays (ELISA) and compared with controls not treated with candidate compounds, and controls not stimulated with VEGF, respectively.

The ELISA for detection and measure of the degree of tyrosine kinase activity may generally be conducted according to protocols known in the art, which are described in, for example, Voller *et al.*, 1980, "Enzyme-Linked Immunosorbent Assay," In: *Manual of Clinical Immunology, 2d ed.*, edited by Rose and Friedman, pp 359-371 (Am. Soc. Of Microbiology, Washington, D.C.) and in the co-pending U.S. Patent

Application No. 08/279,321, filed July 22, 1994, incorporated herein by reference in its entirety.

For example, the ELISA plates may be coated with a suitable antibody against KDR/FLK-1, such as L4 or E38, *see*, Section 6.7. Lysates of stimulated and non-stimulated cells are added and incubated under suitable conditions. After washing, the ELISA plates are incubated with anti-phosphotyrosine antibody, linked to a detection entity, *e.g.*, biotin. In the specific embodiments, the biotinylated anti-phosphotyrosine antibody 4G10 (UBI, Catalog No. 16-103) is used. The plates are washed, the assay developed, and the phosphotyrosine levels of the different wells determined on an ELISA reader, for example a Dynatech MR5000.

Alternatively, the order of the antibodies may be reversed: Anti-phosphotyrosine antibody may be used as a first antibody for coating the ELISA plate, and antibodies against KDR/FLK-1, linked to a detection entity, for example biotin, may be used as the second antibody.

The first assay round serves to quickly eliminate inactive compounds reducing the time and cost of the process. Those subset candidate compounds which inhibit the tyrosine phosphorylation of KDR/FLK-1 by 50% compared to a control are identified and retested using the same assay, however, in the second round the cells are incubated with various concentrations of the subset candidate compounds to determine the $IC_{50}$ value.

In order to identify compounds selectively inhibiting KDR/FLK-1, parallel assays may be performed to determine the subset candidate compounds' effect on the ligand-induced autophosphorylation of NIH 3T3 cells transfected with a control receptor tyrosine kinase, for example the EGF-R or the IGF-R.

Subset candidate compounds with an $IC_{50}$ <50 $\mu$M, preferably <10 $\mu$M, and with a twofold, preferably fivefold selectivity for inhibition of KDR/FLK-1 relative to the control receptor are determined "positive" and further pursued. Test compounds which do not meet this standard are removed.

### 5.1.2. Bioresponse Assay For The Determination Of The Inhibitor's Efficacy In A Biological System

As a second "filter" step, a bioresponse assay is employed to determine the efficacy of the KDR/FLK-1 inhibitors in a more physiologically relevant manner, herein the effect on cultured endothelial cells. A number of endothelial cell lines have been described in the literature which may be employed for this assay. For example, bovine aortic endothelial (BAE) cells and human umbilical vein endothelial (HUV-EC) cells have been shown to express the KDR/FLK-1 receptor as well as a suitable control receptor, the receptor for aFGF. If such cultured endothelial cells are starved by growth factor depletion, a mitogenic effect may be induced and measured upon stimulation with VEGF. A similar mitotic response may be induced by aFGF.

In a preferred embodiment, HUV-EC cells are seeded in tissue culture flasks and grown to a suitable density. After starving, *i.e.*, depletion of growth factors, the cells are incubated with different concentrations of subset candidate compounds. Subsequently, the cells are stimulated with VEGF or aFGF, respectively, and the induced mitogenic effect is measured by determination of, for example, $^3$H-thymidine incorporation into the DNA. The subset candidate compounds' selectivity for inhibition of the VEGF-induced bioresponse is determined by comparison

with the subset candidate compounds' effect on the AFGF-induced bioresponse.

Subset candidate compounds with an $IC_{50}$ of <50 $\mu$M, preferably <10 $\mu$M, and with a selectivity of inhibition against KDR/FLK-1 of twofold, preferably threefold relative to the control, are determined to be "positive" and are further pursued. The "negative" test compounds removed.

### 5.1.3. Biochemical Assay For The Determination Of The Specificity Of Inhibition The Subset Candidate Compound For KDR/FLK-1 Receptor On The Molecular Level

As an additional "filter", a defined *in vitro* assay may be employed to confirm that the inhibitory effects of the subset candidate compounds determined in the first filter cellular assay described in Section 5.1.1. are due to direct interaction of the subset candidate compound with KDR/FLK-1 rather than the result of secondary effects occurring in the cells. Specifically, a biochemical assay may be employed to test the subset candidate compounds' effect on the VEGF-induced autophosphorylation of isolated KDR/FLK-1 *in vitro*. The biochemical assay is an optional step in the assay cascade of this invention; it is simply designed to confirm that KDR/FLK-1 is in fact the direct molecular target of the inhibitor.

For example, NIH 3T3/FLK-1 cells, or any other cultured cells expressing KDR/FLK-1, are lysed, and aliquots of the lysates are distributed on ELISA plates which have been coated with KDR/FLK-1-specific antibodies. After incubation for a suitable time under suitable conditions, the plates are washed to remove the unbound proteins and the subset candidate compounds are added to the wells. Subsequently, the

receptor kinase reaction is induced by addition of kinase buffer containing $MnCl_2$. $Mn^{2+}$ ions are known to stimulate enzymatic activity of receptor tyrosine kinases and thus receptor autophosphorylation. The plates are then washed, and incubated with an anti-phosphotyrosine antibody, linked to a detection entity, *e.g.*, biotin. In preferred embodiments, biotinylated anti-phosphotyrosine antibody 4G10 is used (UBI, Catalog No. 16-103). The plates are washed, the assay developed, and the phosphotyrosine levels of the different wells determined on an ELISA reader, for example a Dynatech MR5000. Subset candidate compounds determined to inhibit the *in vitro* autophosphorylation are further pursued, the "negative" ones are removed.

As the biochemical assays, as true for the cellular assay described herein, may be adopted as a initial high-throughput screening assay, the biochemical assay may employed in a ddition of, or as substitute of, the cellular assay described as the "first filter". Preferably, however, both assays are used to verify the selectivity and potency of a test compound for KDR/FLK-1 as the molecular target on two independent assay levels.

### 5.1.4. Determination Of Toxicity Of The Compounds

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. The tolerance of the subset candidate compounds may be assessed by determination by assessment of the $LD_{50}$ value, *i.e.*, the lethal dose for 50% of a group of animals. Various doses of the test compound, estimated to cover the range from 0 to 100% lethality, are administered to several groups of animals. The mortality in each

group within a fixed period of time is determined and used to construct a curve relating factual mortality to dose. Determination of $LD_{50}$ values is further described in Fabian *et al.*, 1993, *Regulatory Toxicology and Pharmacology 18*:206-213; Paumgartten *et al.*, 1989, *Brazilian J. Med. Biol. Res. 22*:987-991.

### 5.1.5. Determination Of The Compounds Efficacy *In Vivo* In A Mouse Subcutaneous Xenograft Tumor Model

The subset candidate compounds which have been determined to effectively and selectively inhibit the signal transduction induced by VEGF and mediated by KDR/FLK-1 are further analyzed in an *in vivo* model relevant to the process of vascularization and/or angiogenesis. In general, suitable models include, but are not limited to, *in vivo* tumor models, tumor invasion models, retinopathy models, etc.

In a specific emdodiment of the invention, a mouse xenograft tumor model is employed as angiogenesis model. In general, tumor cell lines are implanted subcutaneously in nude mice. Subsequently, the animals are treated with the candidate compounds to determine their effect on tumor angiogenesis and tumor growth. In general, maximum dose of compound which is administered to the animal is the amount which has been determined as the $LD_{10}$ value, *i.e.*, the dose lethal for 10% of the test animals. The xenografts are measured regularly, preferably at least every three days. At the end of the experiment, the tumors are resected to be immunologically and histologically examined to determine of the subset candidate compounds' inhibitory effect on the tumor angiogenesis.

In a specific embodiment of the invention, EPH-4 (Reichmann *et al.*, 1989, *J. Biol. Cell. 108*:1127-1138) cells transfected with VEGF (EPH-4/VEGF cells) are

employed as test tumors. Wild type EPH-4 cells are immortalized epithelial cells which do not grow as tumor when implanted subcutaneously in nude mice. EPH-4/VEGF cells which have been engineered to express high levels of VEGF, however, have been shown to grow as highly vascularized subcutaneous tumors in test animals. In contrast to other tumor cells, which produce VEGF only after induction, for example by hypoxia and the hypoxia induced factor (Liu *et al.*, 1995, *Circ. Res.* 77:638-643), EPH4/VEGF cells produce VEGF constitutively. Accordingly, they are extremely potent inducers of tumor angiogenesis and as such provide for a very stringent *in vivo* test system.

Those subset candidate compounds which are determined to inhibit the EPH-4/VEGF tumor growth by at least 30% relative to untreated controls at a doses smaller than the $LD_{10}$, are the desired compounds of this invention.

### 5.1.6. Summary Of The Experimental Results

TABLE I summarizes the compounds identified in each "filter" of the assay cascade of the invention.

TABLE I
COMPOUND SELECTION THROUGH ASSAY CASCADE COMPRISING FILTERS OF INCREASING STRINGENCY

| SUGEN I.D. | CELLULAR ASSAY | | BIORESPONSE ASSAY | | *IN VIVO* EFFICACY | BIOCHEMICAL ASSAY |
|---|---|---|---|---|---|---|
| | $IC_{50}$($\mu$M) | Selectivity | $IC_{50}$($\mu$M) | Selectivity | | ($\mu$M) |
| SU5416 | 0.1 | >1000 | 0.04 | 1250 | yes | |
| SU4312 | 0.8 | >125 | 0.8 | >62.5 | yes | 0.5 |
| SU4932 | 2 | >50 | 2.5 | 18.1 | yes | 4.8 |
| SU4943 | 7.6 | >13.1 | 3.1 | 5.7 | yes | 3.6 |
| SU5208 | 4.7 | >21.3 | 9.3 | >5.3 | no | |
| SU4314 | 1 | >100 | 0.2 | 30 | no | 0.01 |
| SU4928 | 8.7 | >11.5 | 3.9 | 3.7 | no | 3.3 |
| SU4929 | 6 | >16.7 | 3.8 | >13.2 | no | 1.6 |
| SU1385 | 2.3 | >43 | 0.3 | 52.3 | no | 0.02 |
| SU4304 | 9 | 5.8 | 7.2 | 1.07 | | |
| SU4334 | 9.7 | 6.4 | 14.4 | 1.0 | | |
| SU0879 | 0.8 | 26 | 0.6 | 1.3 | | |
| SU4161 | 0.5 | 52 | 2.1 | 1.0 | | |
| SU1076 | 9.2 | >10 | 10 | 1.8 | | |
| SU1433 | 9.3 | >11 | 7 | 0.8 | | |
| SU4348 | 8 | >12.5 | 7.6 | 1.05 | | |
| SU1498 | 0.7 | >143 | 2.5 | 1.0 | | |
| SU4945 | 5 | >20 | 3.1 | 6.1 | | 5.4 |
| SU4157 | 4 | >25 | 5.3 | 1.04 | | |
| SU4136 | 1.8 | >28 | 8 | 1.03 | | |
| SU1835 | 3.4 | >29 | 30 | 1.0 | | |
| SU4328 | 2.8 | >35.7 | 2.9 | 1.0 | | |
| SU4209 | 0.7 | >71 | 2.5 | 2.6 | | |
| SU5015 | 10 | 0.57 | | | | |
| SU4936 | 8.5 | 1.1 | | | | |
| SU5014 | 9.9 | 1.3 | | | | |
| SU1387 | 4.9 | 1.9 | | | | |
| SU4313 | 4 | 2.75 | | | | |
| SU1393 | 3.3 | 4.8 | | | | |

### 5.2. Source Of Compounds

The test candidate compounds employed for the process of this invention may be obtained from any commercial source, including Aldrich (1001 West St. Paul Ave., Milwaukee, WI 53233), Sigma Chemical (P.O. Box 14508, St. Louis, MO 63178), Fluka Chemie AG (Industriestrasse 25, CH-9471 Buchs, Switzerland (Fluka Chemical Corp. 980 South 2nd Street, Ronkonkoma, NY 11779)), Eastman Chemical Company, Fine Chemicals (P.O Box 431, Kingsport, TN 37662), Boehringer Mannheim GmbH (Sandhofer Strasse 116, D-68298 Mannheim), Takasago (4 Volvo Drive, Rockleigh, NJ 07647), SST Corporation (635 Brighton Road, Clifton, NJ 07012), Ferro (111 West Irene Road, Zachary, LA 70791), Riedel-deHaen Aktiengesellschaft (P.O. Box D-30918, Seelze, Germany), PPG Industries Inc., Fine Chemicals (One PPG Place, 34th Floor, Pittsburgh, PA 15272). Further any kind of natural products may be screened using the assay cascade of the invention, including microbial, fungal or plant extracts.

### 5.3. Compounds Identified Using The Process Of The Invention

Using the process of this invention, a number of compounds selectively targeting and inhibiting the signal transduction induced by VEGF and mediated by KDR/FLK-1 for the inhibition of vasculogenesis and/or angiogenesis *in vivo* have been identified. The compounds identified by the process of the invention include, for example, derivatives of quinazoline, quinoxiline, substituted aniline, indoline, isoxazoles, acrylonitrile and phenylacrylonitrile compounds. The compounds of the present invention, however, are not limited to any particular chemical structure, as they are solely defined by the assay

cascade of the invention, which allows, for the first time, to systematically and rationally identify highly potent and highly selective inhibitors targeting the activity of KDR/FLK-1 on a molecular level.

In addition to the above compounds and their pharmaceutically acceptable salts, the invention is further directed, where applicable, to solvated as well as unsolvated forms of the compounds (*e.g.* hydrated forms) produced and identified by the process of the invention.

**5.4. Indications**

The compounds identified by the methods of the present invention are believed to bind to and specifically inhibit the KDR/FLK-1 receptor by, *e.g.*, inhibiting its catalytic activity, affinity for ATP or ability to interact with a substrate.

Thus, pharmaceutical compositions comprising a therapeutically effective amount of a compound identified by the process of the invention will be useful for the treatment of diseases driven by persistent unregulated angiogenesis. For example, in arthritis, new capillary blood vessels invade the joint and destroy the cartilage. Further, in at least twenty (20) diseases relating to ocular neovascularization, as for example diabetes mellitus, new and bleeding capillaries cause blindness.

Moreover, vasculogenesis and/or angiogenesis has been associated with the growth of malignant solid tumors and metastasis. Indeed, a tumor must continuously stimulate the growth of new capillary blood vessels for the tumor itself to grow. Furthermore, the new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation and to metastasize to distant sites in the body. Thus, the compounds and pharmaceutical

compositions identified by the process of the present invention may be included in methods for treating, among other diseases, diabetic retinopathy and other diseases related to ocular neovacularization, arthritis, glioma, melanoma, Kaposi's sarcoma, psoriasis, hemangioma and ovarian, breast, lung, pancreatic, prostate, colon and epidermoid cancer.

Thus, in general, the disorders which may be treated with the compounds and compositions, and pharmaceutical formulations identified by the process of the invention generally refer to angiogenic and vasculogenic disorders resulting in or caused by inappropriate proliferation of blood vessels.

### 5.5. Pharmaceutical Formulations And Routes Of Administration

The identified compounds can be administered to a human patient alone or in pharmaceutical compositions where they are is mixed with suitable carriers or excipient(s) at therapeutically effective doses to treat or ameliorate a variety of disorders. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms as determined in a decrease of vasculogenesis and/or angiogenesis. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

#### 5.5.1. Routes Of Administration.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct

intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer a compound of the invention in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot, or in a sustained release formulation.

Furthermore, one may administer the drug via a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

### 5.5.2. Composition/Formulation

The pharmaceutical compositions of the present invention may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained as a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients include fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The

push-fit capsules can contain the active ingredients in admixture with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration,the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin, for use in an inhaler or insufflator, may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, such as sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase.

The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone; and other sugars or polysaccharides may be substituted for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually with a greater toxicity.

Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the KDR/FLK-1 inhibiting compounds of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

### 5.5.3. Effective Dosage.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For

example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the $IC_{50}$ as determined in cell culture (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of the KDR/FLK-1 activity). Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between $LD_{50}$ and $ED_{50}$. Compounds which exhibit high therapeutic indices are preferred.

The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the $ED_{50}$ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (*See, e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase

modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; *e.g.*, the concentration necessary to achieve 50-90% inhibition of the kinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

### 5.5.4. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the label may include inhibition of

angiogenesis, treatment of a tumor, treatment of arthritis, diabetes, and the like.

## 6. EXAMPLES

The following assays and animal models may be used to identify compounds which selectively inhibit the KDR/FLK-1 receptor signal transduction. More specifically, high-throughput cellular and biochemical assays are designed for the preselection of subset candidate compounds having inhibitory activity on KDR/FLK-1. Subset candidate compounds identified as positive by these "filters" are then evaluated in bioresponse assays and animal models for their selectivity for inhibition of VEGF-induced and KDR/FLK-1-mediated signal transduction and for their *in vivo* efficacy.

### 6.1. Cellular Assays For The Initial Screening Of The Acquired Test Compounds

The following assay has been developed to provide for the high throughput testing of test candidate compounds for the identification of potent KDR/FLK-1-specific inhibitors. NIH 3T3 cells expressing FLK-1 (NIH 3T3/FLK-1 cells) are seeded and grown on 96 well plates and incubated with the test candidate compounds or extracts. Control cells, *e.g.*, NIH 3T3 cells expressing EGF-R (NIH 3T3/EGF-R) are treated identically. The cells are stimulated with the corresponding ligand, VEGF and EGF, respectively, lysed, and receptor tyrosine phosphorylation is determined via Enzyme Linked Immunosorbent Assays (ELISA).

In general, the ELISA for detection and measurement of the presence of tyrosine kinase activity may be conducted according to protocols known in the art, which are described in, for example,

Voller *et al.*, 1980, "Enzyme-Linked Immunosorbent Assay," In: *Manual of Clinical Immunology, 2d ed.*, edited by Rose and Friedman, pp 359-371 Am. Soc. Of Microbiology, Washington, D.C. The objective of the following protocol is to provide a consistent method for measuring phosphotyrosine levels of FLK-1 receptors isolated from lysates of FLK-1/NIH cells.

Selectivity of inhibition of the subset candidate compounds for KDR/FLK-1 relative to control receptor (here the EGF-R) is determined. Compounds having an $IC_{50}$ of <50 $\mu$M, preferably <10 $\mu$M, and further a selectivity of twofold, preferably fivefold, for KDR/FLK-1 are "positive" and are further pursued

**6.1.1. Reagents And Supplies**

1. Corning 96-well ELISA plates (Corning, Catalog No. 25805-96).

2. PBS (Phosphate Buffered Saline); Formulation: 2.7 mM KCI; 1.1 mM $KH_2PO_4$; 0.5 mM $MgCl_2$ (anhydrous); 138 mM NaCl; 8.1 mM $Na_2HPO_4$.

3. HNTG Buffer; Formulation: 20 mM ptkHEPES buffer pH 7.5; 150 mM NaCl; 0.2% Triton X-100; 10% Glycerol.

4. EDTA (0.5 M pH 7.0).

5. $Na_3VO_4$ (0.1 M pH 10.0).

6. $Na_4P_2O_7$ (0.2 M).

7. DMEM (Dulbecco's Modified Eagle Medium) with 1x high glucose, L-Glutamine (Catalog No. 11965-050).

8. FBS (Fetal Bovine Serum); CS (Calf Serum).

9. L-Glutamine (200 mM stock).

10. Growth media: DMEM 10% heat inactivated FBS (10% CS)+ 2 mM L-Glutamine.

11. Starvation media: DMEM 0.1% FBS (0.1% CS)+ 2 mM L-Glutamine.

12. NIH 3T3/FLK-1 cells, NIH 3T3/EGF-R cells, grown in growth media in 5% $CO_2$ at 37°C.

13. VEGF: 10 μg/ml in Milli-Q water stored at -20°C (Peprotech, -20°C).

14. EGF: stock concentration = 16.5 μM; EGF 201, TOYOBO, Co., Ltd. Japan.

15. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

16. Anti-FLK-1D monoclonal antibody: Produced and purified by the Biochemistry laboratory, Sugen Inc.

17. 4G10 Biotin-conjugated anti-phosphotyrosine (UBI, Catalog No. 16-103).

18. Solution A+B: (Vector Laboratories, Burlingame, California, Catalog No. PK-6100.)

19. ABTS solution; Formulation: 100 mM Citric acid (anhydrous); 250 mM $Na_2HPO_4$, pH 4.0; 0.5 mg/ml ABTS (2,2-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid). Keep solution in dark at 4°C until ready to use.

20. Hydrogen peroxide 30% (Fisher, Catalog No. H325).

21. ABTS/$H_2O_2$; Formulation: 15 ml ABTS solution; 8 μl $H_2O_2$.

22. 0.1 M $Na_2CO_3$, pH 9.6.

23. TBSW Buffer (Tris buffered Saline with Tween-20); Formulation: 50 mM Tris pH 7.2; 150 mM NaCl; 0.1% Tween 20.

24. 20% Ethanolamine Stock, pH 7.0.

25. Corning 96-well round bottom cell culture plates (Corning, Catalog No. 25850).

26. Nunc Polyproplene 96-well V bottom plates.

27. HNTG*; Formulation: HNTG plus 5 mM $NaVO_4$; 5 mM EDTA; 2 mM $NaP_2O_7$.

28. TBSW/0.5% Ethanolamine; Formulation: TBSW buffer plus 0.5% Ethanolamine.

**6.1.2. Procedure**

***A. Coating And Blocking Of ELISA Plates.***

1. Corning ELISA plates are coated with 4.0 μg FLK-1D or EGF-R (05-101) antibody/well, respectively, in 0.1 M $Na_2CO_3$ at a final volume per well of 100 μl over night at 4°C. Plates can be used for at least three days when kept at 4°C.

2. Unbound antibody is removed from wells by inverting plate. Plates are washed 3x with TBSW/0.5% Ethanolamine.

3. Plates are blocked by incubation with 200 μl TBSW/0.5% Ethanalmine per well while shaking at room temperature for 30 min.

***B. Growth And Seeding Of FLK-1/NIH Cells***

1. NIH 3T3/FLK-1 and NIH 3T3/EGF-R cells, respectively, are plated in a 15 cm dish with 30 ml growth media and grown to 90-100% confluence. Subsequently, cells are harvested by trypsinization (3 ml trypsin/EDTA per 15 cm dish). Trypsinization is stopped by addition of 10 ml growth medium. Then, the cells are sedimented by centrifugation for 5 min at 2,000x g, and the cells resuspended in growth media to yield a final dilution of 25,000 cells/100 μl.

2. NIH 3T3/FLK-1 and NIH 3T3/EGF-R cells, respectively, are seeded into Corning 96 cell plate at 25,000 cells/well in 100 μl growth media.

3. Cells are grown for 1-2 days at 37°C, 5% $CO_2$.

4. Cells are washed with 100 μl of PBS per well.

5. The PBS is removed and 100 μl of Starvation medium are added to each well. The cells are incubated overnight at 37°C, 5% $CO_2$.

*C. **Assay Procedure.***

1. The compound/extract stocks at a concentration of about 10mM are diluted 1:10 in a polypropylene 96-well plate using DMEM. DMSO is diluted 1:10 for control wells.

2. The starvation media is removed from the wells of the cell plates and 90 $\mu$l of DMEM are added to each well.

3. 10 $\mu$l of diluted compound/extract and controls are added to the wells. The final drug dilution and DMSO dilution is 1:100.

4. The cells are incubated with diluted compounds/extracts for 2 hours at 37°C, 5% $CO_2$.

5. NIH 3T3/FLK-1 cells are stimulated with 50 $\mu$l/well of 3 mM $Na_3VO_4$ and 0.3 $\mu$l/ml VEGF in DMEM (final concentration of 1 mM $Na_3VO_4$ and 100 ng/ml VEGF) for 8 minutes at 37°C. Control NIH 3T3/FLK-1 cells are incubated with 50 $\mu$l/well of 3 mM $Na_3VO_4$ only. NIH 3T3/EGF-R cells are stimulated with EGF (and optionally with $Na_3VO_4$) at a final concentration of 25 nM (and 1 mM), respectively. Control NIH 3T3/EGF-R cells are incubated with 50 $\mu$l/well of 3 mM $Na_3VO_4$ accordingly.

6. After incubation with/without the respective ligands, the supernatants are aspirated and the cell plates washed 1x with PBS.

7. Cells are lysed in 100 $\mu$l of HNTG* by incubation on ice for 5 minutes.

8. The ELISA plates are washed 3x as described above in step A.2.

9. The lysates of stimulated and non-stimulated cells are transferred from cell plates to the corresponding wells of the ELISA plates by repeated aspiration and dispensing while scraping the sides of each cell well. The ELISA plates are

incubated with the lysates for 2 hours at 4°C while shaking.

10. The ELISA plates are washed 3x as described in step A.2.

11. 100 µl/ biotinylated 4G10, diluted 1:10000 in TBSW/0.5% Ethanolamine are added to each well and incubated for 30 minutes while shaking.

12. At the same time as step 11, A+B reagents are mixed at a dilution of 1:5000 in TBSW/0.5% Ethanolamine. The solution is incubated at room temperature until step 13 is completed.

13. The ELISA plates are washed 3x as described in step A.2..

14. Per well, 100 µl of the A+B reagent mixture are added and incubated while shaking for 30 minutes.

15. The ELISA plates are washed 3x with TBST and twice with water.

16. ABTS/$H_2O_2$ solution is prepared 5 minutes prior to use.

17. The ELISA plate is developed by addition of 100 µl/well of the ABTS/$H_2O_2$ solution. The plates are incubated for approximately 10 minutes and then read on a Dynatech MR5000 at 410 nm against a reference filter of 630 nm.

### 6.1.3. Experimental Results From Cellular Assays

Subset candidate compounds obtained with the cellular assay are summarized in TABLE II.

**TABLE II**
**SELECTIVITY AND POTENCY OF COMPOUNDS IDENTIFIED IN HIGH-THROUGHPUT CELLULAR ASSAY**

| SUGEN I.D. | FLK-1R Kinase $IC_{50}$ ($\mu$M) | EGFR Kinase $IC_{50}$ ($\mu$M) | Specificity | |
|---|---|---|---|---|
| SU4304 | 9 | 52 | 5.8 | |
| SU4334 | 9.7 | 62 | 6.4 | |
| SU0879 | 0.8 | 21 | 26 | |
| SU4161 | 0.5 | 26 | 52 | |
| SU1076 | 9.2 | >100 | >10 | |
| SU5208 | 4.7 | >100 | >21.3 | |
| SU4314 | 1 | >100 | >100 | |
| SU5416 | 0.1 | >100 | >1000 | |
| SU1433 | 9.3 | >100 | >11 | |
| SU4928 | 8.7 | >100 | >11.5 | |
| SU4348 | 8 | >100 | >12.5 | |
| SU4312 | 0.8 | >100 | >125 | |
| SU4943 | 7.6 | >100 | >13.1 | |
| SU1498 | 0.7 | >100 | >143 | |
| SU4929 | 6 | >100 | >16.7 | |
| SU4945 | 5 | >100 | >20 | |
| SU4157 | 4 | >100 | >25 | |
| SU4136 | 1.8 | >50 | >28 | |
| SU1835 | 3.4 | >100 | >29 | |
| SU4328 | 2.8 | >100 | >35.7 | |
| SU1385 | 2.3 | >100 | >43 | |
| SU4932 | 2 | >100 | >50 | |
| SU4209 | 0.7 | >50 | >71 | 5 FOLD OR GREATER |
| SU5015 | 10 | 5.7 | 0.57 | |
| SU4936 | 8.5 | 9.5 | 1.1 | |
| SU5014 | 9.9 | 13 | 1.3 | |
| SU1387 | 4.9 | 9.3 | 1.9 | |
| SU4313 | 4 | 11 | 2.75 | |
| SU1393 | 3.3 | 15.8 | 4.8 | |

333333333333333333333333333333333

### 6.2. Biochemical Assay For Determination Of The Compounds Specificity For KDR/FLK-1

The following biochemical assay is designed to confirm a subset candidate compound's inhibitory effect on the KDR/FLK-1 receptor on a molecular level. More specifically, the following assay is designed to measure receptor autophosphorylation on isolated receptors in a well-defined *in vitro* system, where secondary effects/influences of other cellular molecules can be excluded. A subset of candidate compounds obtained from the cellular assar are tested in the biochemical assay. Compounds determined as "positive" in the following assay are compounds which in fact specifically inhibit the autophosphorylation of KDR/FLK-1.

#### 6.2.1. Reagents And Supplies

1. 15 cm tissue culture dishes

2. NIH 3T3/FLK-1 cells: NIH fibroblast line overexpressing human FLK-1 clone 3 Sugen, Inc. (obtained from the Max Planck Institute, Martinsried, Germany).

3. Growth medium: DMEM plus heat inactivated 10% FBS and 2 mM Glutamine, Gibco-BRL, Gaithersburg, USA.

4. Starvation medium: DMEM plus 0.5% heat-inactivated FBS, 2 mM Glutamine (Gibco-BRL, Gaithersburg, USA).

5. Corning 96-well ELISA plates (Corning, Catalog No. 25805-96).

6. L4 or E 38: Monoclonal antibody specific for FLK-1, purified by Protein A-agarose affinity chromatography; Sugen, Inc.

7. PBS (Dulbecco's Phosphate-Buffered Saline), pH 7.2 (Gibco, Catalog No. 450-1300EB). Formulation:

2.7 mM KCl; 1.1 mM $KH_2PO_4$; 0.5 mM $MgCl_2$ (anhydrous); 138 mM NaCl; 8.1 mM $Na_2HPO_4$).

8. HNTG buffer; Formulation: 20 mM Hepes/HCl buffer, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM PMSF, 5 mg/l Aprotinin.

9. Biorad Protein Assay Solution (Biorad, Hercules, CA; Catalog No. 500-0006).

10. Blocking Buffer; Formulation: 5 % carnation instant dry milk in PBS.

11. TBST; Formulation: 50 mM Tris/HCl, 150 mM NaCl, 0.1% Triton X-100.

12. Kinase Buffer; Formulation: 25 mM Hepes/Cl pH 7.0, 100 mM NaCl, 10 mM $MnCl_2$ and 2 % Glycerol.

13. Stop solution: 50 mM EDTA.

14. Biotinylated 4G10, specific for phosphotyrosine (UBI, Lake Placid, NY; Catalog No. 16-103).

15. ABC kit (Vector Laboratories, Burlingame, CA; Catalog No. 4000).

16. DMSO (Sigma, Catalog No. D-2650).

17. NUNC 96-well V bottom polypropylene plates for compounds (Applied Scientific, Catalog No. AS-72092).

18. ABTS Solution; Formulation: 100 mM Citric Acid (anhydrous); 250 mM $Na_2HPO_4$ pH 4.0; 0.5 mg/ml (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, Catalog No. A-1888). (Solution should be kept in the dark at 4°C until ready to use.)

19. Hydrogen peroxide, 30% solution (Fisher, Catalog No. H325). (Should be stored in the dark at 4°C until ready to use.)

20. ABTS/$H_2O_2$; Formulation: 15 ml ABTS solution and 2 µl $H_2O_2$. Prepare 5 minutes before use and leave at room temperature.

21. ATP (Sigma, Catalog No. A-7699).

**6.2.2. Procedures**

***A. Cell Growth And Lysate Preparation.***

1. Cells are seeded into growth medium and grown for 2-3 days to 90-100% confluency at 37°C and 5% $CO_2$. Cells should not exceed passage No. 20.

2. The medium is removed and the cells washed twice with PBS, then lysed with HNTG lysis buffer. All lysates are collected and votexed for 20 seconds.

3. The soluble material is removed by centrifugation (5 min, 10,000x g).

4. The protein concentration is determined according to Bradford.

5. The lysates are divided into 1 mg aliquots and the tubes quick-freezed in a dry ice/ethanol mixture. The tubes are stored at -80°C.

***B. Test Plate And Drug Plate Preparation.***

1. Corning 96-well ELISA plates are coated by incubation with 5 μg/well of purified L4 or E 38 in 100 μl coating buffer overnight at 4°C. Plates can be used for one week when kept at 4°C.

2. The unbound proteins are removed from the wells by inverting the plates to remove the liquid. The plates are patted on a paper towel to remove excess liquid and bubbles.

3. The plates are blocked by incubation with 100 μl blocking buffer per well for 45 minutes while shaking on a microtiter plate shaker.

4. The blocking buffer is removed and the ELISA plates washed three time with TBST. The plates are patted on a paper towel to remove excess liquid and bubbles.

5. The cleared lysate from NIH 3T3/FLK-1 cells are thawed, 75 μg of lysate/well are added, and incubated for 3 hours at 4°C while shaking the plate. Alternatively, the incubation can be performed

overnight, provided the temperature is between 4-0°C. Overnight incubation results in a higher delta in the ELISA.

6. The unbound proteins are removed from wells by inverting the plates. The plates are washed four times with TBST and the plates are patted on a paper towel to remove excess liquid and bubbles.

7. 80 µl of kinase buffer are added to the wells.

8. The compounds (10 mM, dissolved in 100 % DMSO) are diluted 20-fold into wells of a polypropylene plate filled with TBST plus 1% DMSO.

9. 10 µl of the pre-diluted compounds are added to the ELISA wells containing immobilized FLK-1 and mixed. The volume is now 90 µl. Control wells receive no drug.

10. The kinase reaction is started by addition of 10 µl 0.3 mM ATP per well. Negative controls receive distilled water.

11. The plates are incubated for 60 min at room temperature while shaking on a microtiter plate shaker.

12. The liquid is aspirated and the ELISA plate washed four times with TBST. The plate is patted on a paper towel to remove excess liquid.

13. 100 µl of 1:10,000 fold diluted biotinylated 4G10 is added to all wells and the mixture incubate for 45 minutes while shaking the plates. At the same time as the 4G10 addition, solution A+B is added (50 µl each) to 10 ml TBST.

14. The supernatant is aspirated and the plates washed five times as described in step 6.

15. 100 µl of the pre-formed complex (solution A+B in TBST) are added to all wells and incubated for 45 min while shaking the plates.

16. The supernatant is aspirated and the plates are washed five times as described in step 6.

17. 100 $\mu$l of the ABST/$H_2O_2$ solution are added to each well. The plates are incubated 10-15 minutes while shaking the plates.

18. The bubbles are removed using a stream of air.

19. The plates are read on a Dynatech MR5000 ELISA reader at 410 nM against a reference filter of 630 nM.

### 6.2.3. Experimental Results From Biochemical Assays

Subset candidate compounds obtained with the biochemical assay are summarized in TABLE III.

**TABLE III**
**POTENCY OF TARGET SPECIFIC COMPOUNDS IDENTIFIED IN BIOCHEMICAL ASSAY**

| SUGEN I.D. | Inhibition in Biochem FLK-1R Kinase Assay (μM) |
|---|---|
| SU4928 | 3.3 |
| SU4943 | 3.6 |
| SU4945 | 5.4 |
| SU4932 | 4.8 |
| SU4314 | 0.01 |
| SU1385 | 0.02 |
| SU4929 | 1.6 |
| SU4312 | 0.5 |

### 6.3. Determination Of The Bioresponse Via FLK-1 Assay Using HUV-EC-C Cells

The following protocol has been designed to test the efficacy of anti-FLK-1 inhibitors in an *in vitro* assay using a human umbilical vein endothelial cell line (HUV-EC-C). HUV-EC-C express the KDR/FLK-1 receptor and respond to induction with VEGF with cell proliferation, and are therefore an excellent system in order to determine the subset candidate compound's effect on VEGF-induced bioresponse. As aFGF induces proliferation in HUV-EC cells as well, the subset candidate compounds' effect on the aFGF-induced bioresponse is measured in control experiments in order to determine the subset candidate compounds' selectivity inhibition for KDR/FLK-1. Subset candidate compounds inhibiting the VEGF-induced bioresponse of HUV-EC-C at an $IC_{50}$ <10 μM at a threefold selectivity relative to the bioresponse induced by aFGF, are tested further.

**6.3.1. Procedure**

***A. Day 1.***

1. HUV-EC-C cells (human umbilical vein endothelial cells, American Type Culture Collection; catalogue no. 1730 CRL) are washed twice with Dulbecco's phosphate-buffered saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029) and then trypsinized with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company; catalogue no. C-1544). After cells have detached from the flask, they are resuspended in D-PBS and transferred to a 50 ml sterile centrifuge tube (Fisher Scientific; catalogue no. 05-539-6).

2. The cells are sedimented by centrifugation, the supernatant is aspirated. The cells resuspended and washed twice or three times with D-PBS. Finally, the cells are resuspended in about 1 ml assay medium/15 $cm^2$ of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL; catalogue no. 21127-014) + 0.5% heat-inactivated fetal bovine serum. The cells are counted with a Coulter Counter®v Coulter Electronics, Inc.); the cell suspension is diluted in assay medium to the cells to obtain a concentration of $0.8\text{-}1.0x10^5$ cells/ml.

3. The cells are seeded in 96-well flat-bottom plates at 100 µl/well or $0.8\text{-}1.0x10^4$ cells/well and incubated 37°C, 5% $CO_2$ for about 24 hours.

***B. DAY 2.***

1. The compounds are adjusted to 100x working stocks. Typically, the compounds are in 20 mM stocks, however, some are at other concentrations. At 20 mM, the compounds are diluted 1:100 to arrive at 200 µM, which is a 4x concentration (*i.e.*, it comprises 1/4 of the total volume of the well and ultimately will be 50 µM). If the compounds are dissolved in DMSO, they

should not be diluted <1:50, because higher concentrations of DMSO might kill the HUV-EC-C cells.

2. For each candidate compound a total amount 90 μl/well are needed for seven (7) wells, *i.e.*, three (3) well for VEGF and aFGF, respectively, and one (1), well for non-ligand media control.

3. Since the compounds are in 1:100 DMSO:assay medium, a diluent of the same DMSO:assay medium ratio needs to be made for the compound titrations to keep the DMSO concentration constant when diluting the compound.

4. The compounds are titrated as follows:

a. For better results, 96-well round-bottom plates are used to do the compound titrations.

b. 90 μl/well of compound is added per in the well of the top row (row A) of designated plates. In a typical experiment, two compounds can be assayed/plate (3 columns x 2 ligands (VEGF, aFGF) x 2 drugs). In the top well of the columns for control without, 90 μl/well of the compound are added as well.

c. 60 μl/well of the DMSO:assay medium diluent are added to the rest of the rows of the plates (row B-H) where compound had been added to row A.

d. Three-fold dilutions are made by pipetting 30 μl of the 90 μl/well in row A into row B. 30 μl of the 90 μl/well in row B into row C, and so on down to row G. In the end, the additional 30 μl/well in row G are removed. No compounds are added to row H which is left as the no-drug control. At the end of this pipetting cascade, each well A-G should contain 60 μl of compound at different concentrations. For example, if the compound stock solution was 20 mM and diluted 1:100 to 200 μM, giving 50 μM final concentration, the dilutions yield compound

concentrations as follows: 50, 16.6, 5.5, 1.8, 0.6, 0.2, 0.07, and 0 μM.

e. 50 μl/well of the 60 μl per well are transferred to the assay plate and incubated for 2-3 h at 37°C.

5. Ligand (VEGF and aFGF) and media control is added as follows:

a. For each compound tested, 1.2 ml of VEGF and aFGF will be needed (50 μl/well, 24 wells total per compound).

b. For VEGF, the stock is 10 μg/ml, and the final concentration in the assay is 20 ng/ml. As the volume of VEGF is 1/4 that of the total assay volume (as true for the compounds), a 4x concentration is needed (80 ng/ml).

c. For aFGF, the stock is 10 μg/ml (as with VEGF), and the final concentration in the assay is 0.25 ng/ml. Since the volume of aFGF is 1/4 that of the total assay volume (as true for the compounds and VEGF), a 4x concentration is needed (1 ng/ml).

d. 50 μl of VEGF are added to columns 1-3 and 7-9; add 50 μl of aFGF to columns 4-6 and 10-12, except the media control wells, which get 50 μl/ml of assay medium.

e. The plates are incubated overnight (20-24 h) at 37°C.

***C. DAY 3.***

1. 1 μCi $^{3}$H-thymidine/well (10 μl/well of 100 μCi/ml solution stock solution) are added and incubated overnight (20-24 h) at 37°C.

***D. DAY 4.***

1. The plates are frozen overnight at -20°C.

*E. DAY 5.*

1. The plates are thawed and the cells harvested with Tomtec 96-well plate harvester and applied to filter mats. The $^{3}H$-thymidine incorporation is determined using the betaplate liquid scintillation counter.

### 6.3.2. Experimental Results From HUV-EC-C Cell Assays

Subset candidate compounds obtained with the bioresponse assay are summarized in TABLE IV.

**TABLE IV**
**SELECTIVITY AND POTENCY OF COMPOUNDS IDENTIFIED IN BIORESPONSE ASSAY**

| SUGEN I.D. | HUVEC VEGF Thy incorp $IC_{50}$ ($\mu$M) | HUVEC FGF Thy incorp $IC_{50}$ ($\mu$M) | Specificity Factor | |
|---|---|---|---|---|
| SU4928 | 3.9 | 14.5 | 3.7 | |
| SU4943 | 3.1 | 17.7 | 5.7 | |
| SU4945 | 3.1 | 18.8 | 6.1 | |
| SU4932 | 2.5 | 45.2 | 18.1 | |
| SU4314 | 0.2 | 6 | 30 | |
| SU1385 | 0.3 | 15.7 | 52.3 | |
| SU5416 | 0.04 | 50 | 1250 | |
| SU4929 | 3.8 | >50 | >13.2 | |
| SU5208 | 9.3 | >50 | >5.3 | |
| SU4312 | 0.8 | >50 | >62.5 | 3 FOLD OR GREATER |
| SU1433 | 7 | 5.6 | 0.8 | |
| SU4136 | 8 | 8.2 | 1.03 | |
| SU4157 | 5.3 | 5.5 | 1.04 | |
| SU4348 | 7.6 | 8.3 | 1.05 | |
| SU4304 | 7.2 | 7.7 | 1.07 | |
| SU0879 | 0.6 | 0.8 | 1.3 | |
| SU1076 | 10 | 18.3 | 1.8 | |
| SU4209 | 2.5 | 6.5 | 2.6 | |
| SU1498 | 2.5 | 2.6 | 1.0 | |
| SU1835 | 30 | 31 | 1.0 | |
| SU4161 | 2.1 | 2.0 | 1.0 | |
| SU4328 | 2.9 | 3.0 | 1.0 | |
| SU4334 | 14.4 | 14.6 | 1.0 | |

### 6.4. *In Vivo* Animal Models

All subset candidate compounds which have been determined to effectively and selectively inhibit the

signal transduction induced by VEGF and mediated by KDR/FLK-1 are further analyzed in an *in vivo* mouse xenograft tumor model.

More specifically, EPH-4 cells transfected with VEGF (EPH-4/VEGF cell) propagated in tissue culture flasks. 3x $10^6$ cells in PBS are implanted in 100 $\mu$M subutaneously in the hind flank of nude mice. Dosing of subset candidate compounds is initiated on the following day with daily i.p. injections (in 50 - 100 $\mu$l). The growth of the implanted tumor cells is monitored and the size determined twice a week using calipers. Subset candidate compounds which inhibit tumor angiogenesis *in vivo* as indicated by inhibition of the tumor growth by at least 30% relative to untreated controls, at a doses smaller than the $LD_{10}$ are identified. At the end of the experiment, the tumors are resected and further examined immunologically and histologically to determine the candidate compounds' inhibitory effect on tumor angiogenesis.

#### 6.4.1. Experimental Results From The Mouse Subcutaneous Xenograft Assays

Subset candidate compounds obtained with the subcutaneous xenograft model are summarized in TABLE V.

**TABLE V**
***IN VIVO* EFFICACY OF COMPOUNDS**

| SUGEN I.D. | Efficacy |
|---|---|
| SU4943 | yes*** |
| SU4932 | yes*** |
| SU5416 | yes*** |
| SU4312 | yes*** |
| SU4928 | no |
| SU4929 | no |
| SU5208 | no |
| SU4945 | * |
| SU4314 | no |
| SU1385 | no |

* not tested

*** > 30% inhibition

**6.5. Tumor Invasion Model**

The following tumor invasion model has been developed and may be used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor by the cascade of screening assays of the invention.

**6.5.1. Procedure**

8 week old nude mice (female) (Simonsen Inc.) was used as experimental animals. Implantation of tumor cells was performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject $10^7$ tumor cells in a volume of 100 $\mu$l medium. The cells are injected either into the

duodenal lobe of the pancreas or in the serosa of the colon. The peritoneum and muscles are closed with a 5-0 silk continuous suture and the skin by gluing with Vetbond and with at least two (5-0 silk) interrupted sutures. Animals are observed daily.

#### 6.5.2. Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases, to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurements of tumor size, grade of invasion, immunochemistry, and in situ hybridization).

### 6.6. Production Of FLK-1-Specific Monoclonal Antibodies

***A. Antigen Production.***

A fragment encoding for the 523 bp C-terminal fragment of FLK-1 was constructed into a pGEX3X vector. XL1-Blue Electroporation-competent cells (Stratagene No. 200228) were transformed with the "FLK-1D-GST(pGex-3X FLK-1-523(D)) construct by electroporation. Expression was induced by IPTG induction. The fusion protein was purified to homogeneity by Glutathione agarose affinity chromatography, then the purified protein dialyzed against PBS, concentrated by ultra filtration and stored at - 80°C.

***B. Immunization.***

Balb C mice were immunized with the above described antigen according to standard procedures. *See*, Harlow and Lane, 1988.

*C. Antisera Testing.*

After the initial immunization and boosts with antigen, the mice were bled and the serum tested in the FLK-1 D-GST ELISA.

*D. FLK-1 D-GST-ELISA.*

Purified FLK-1 D-GST or GST control alone were coated to ELISA plates according to standard procedures. Antisera were tested for cross-reactivity with antigen by incubating serially diluted antisera with antigen, followed by HRP-conjugated Goat-anti mouse antiserum.

*E. Fusion.*

Splenocyte/Myeloma cell fusion was performed and hybridoma cells were cultured according to standard procedures.

*F. Screening Of Supernatants.*

Supernatants were screened using the FLK-1D-GST ELISA. Pools that tested positive in the ELISA were subcloned. In the next round of screening, only pools were selected, which cross-related with FLK-1D-GST and not with GST-controls.

*G. Testing Hybridoma Supernatants With Full Length FLK-1 In Western Blotting.*

Positive pools were tested in Western blotting using lysates from NIH 3T3/FLK-1 clone 3 (NIH 3T3 cells transfected with FLK-1). Pools which specifically recognized the FLK-1 receptor protein were further subcloned.

***H. Testing Hybridoma Supernatants With Full Length FLK-1 In Immunoprecipitation.***

Positive pools were tested in immunoprecipitation using lysates from NIH 3T3 cells/FLK-1 clone 3. Positive pools were further subcloned. All clones were tested in immunoprecipitation experiments comparing lysates from non-stimulated vs. VEGF-stimulated NIH 3T3/FLK-1 cells. Western blots were probed with anti-phosphotyrosine specific antiserum. Immunoprecipitates from all clones showed ligand dependent tyrosine phosphorylation of FLK-1.

***I. Final Evaluation Of FLK-1 Specific Monoclonal Antibodies.***

Antibodies from FLK-1 specific clones were purified by Protein A/G agarose affinity chromatography according to standard procedures.

Purified antibodies were coated to ELISA plates and were tested for the ability to capture FLK-1 from lysates of VEGF-stimulated and non-stimulated NIH 3T3/FLK-1 cells. Clones were obtained which fulfilled these criteria (*eg.*, L4 and E 38).

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein are hereby incorporated by reference in their entirety.

## CLAIMS

What Is Claimed Is:

1. A process for producing a compound that inhibits vasculogenesis and/or angiogenesis comprising screening a plurality of test compounds to identify a subset of compounds which selectively and potently inhibits the VEGF receptor.

2. A process for identifying highly potent and selective compounds that are specific for inhibition of angiogenesis comprising increasingly selective assays:

(a) performing a cellular assay with a plurality of test compounds to identify a subset of candidate compounds targeting a VEGF receptor, each having an $IC_{50}$ <50 $\mu$M while having an at least twofold $IC_{50}$ for inhibiting a control receptor;

(b) performing a bioresponse assay with said subset of candidate compounds identified in step (a) to identify a subset of candidate compounds having an $IC_{50}$ <50 $\mu$M for inhibiting a bioresponse induced by VEGF while having an at least twofold higher $IC_{50}$ for inhibiting the bioresponse induced by non-VEGF ligand; and

(c) performing an *in vivo* experiment with said subset of candidate compounds identified in step (b) to identify a subset of candidate compounds inhibiting the growth of a subcutaneous tumor *in vivo* at least 30% compared to a control.

3. The process of Claim 2, wherein said process further comprises a defined *in vitro* assay to identify a subset of candidate compounds directly targeting said VEGF receptor, wherein said targeting is determined by inhibition of said VEGF receptor's activity by at least 50% compared to a control.

4. The process of Claim 2 wherein said process comprises:

(a) performing a cellular assay comprising:

(i) incubating cells expressing said VEGF receptor with test candidate compounds at various concentrations to promote inhibition;

(ii) exposing said cells to VEGF;

(iii) determining a subset of candidate compounds exhibiting inhibition of said VEGF receptor compared to a control;

(iv) determining the selectivity of inhibition of said VEGF receptor of each said subset of candidate compounds by repeating steps (i) to (iii) wherein the cells expressing VEGF receptor are substituted by cells expressing a control receptor tyrosine kinase and VEGF in step (ii) is substituted by a high affinity ligand for said control receptor tyrosine kinase; and

(v) identifying a subset of candidate compounds each having an $IC_{50}$ <50 $\mu$M for inhibiting a VEGF receptor and an at least twofold higher $IC_{50}$ for inhibiting a control receptor;

(b) performing a bioresponse assay comprising:

(i) incubating cultured endothelial cells with various concentrations of said subset of candidate compounds identified in step (a)(vi);

(ii) exposing said cultured endothelial cells to VEGF or a non-VEGF control ligand;

(iii) determining the bioresponse induced by VEGF or the non-VEGF control ligand; and

(iv) identifying a subset of candidate compounds having an $IC_{50}$ <50 $\mu$M for inhibiting the bioresponse induced by VEGF and an at least twofold $IC_{50}$ for inhibiting the bioresponse induced by the non-VEGF control ligand; and

(c) performing an *in vivo* experiment comprising:

(i) implanting cells subcutaneously in an animal to induce the growth of a subcutaneous tumor;

(ii) treating said animal with said subset of candidate compounds identified in step (b)(vi);

(iii) determining the growth of said subcutaneous tumor of the treated animals compared to an untreated control;

(iv) identifying the compound inhibiting the growth of said subcutaneous tumor at least 30% compared to said control.

5. The process of Claim 4, wherein step (a) is replaced by:

(a) incubating cells expressing said d VEGF receptor with candidate compounds to promote inhibition;

(b) exposing said cells to VEGF;

(c) determining a subset of candidate compounds exhibiting 50% inhibition of said VEGF receptor compared to a control;

(d) repeating steps (a) to (c) with said subset of candidate compounds selected in step (c), whereby step (a) is modified in that the cells are incubated with various concentrations of said subset of candidate compounds to determine $IC_{50}$ values;

(e) determining the selectivity of inhibition of said VEGF receptor of each said subset of candidate compounds by repeating steps (a) to (c) wherein the cells expressing VEGF receptor are substituted by cells expressing a control receptor tyrosine kinase and VEGF in step (b) is substituted by a high affinity ligand for said control receptor tyrosine kinase; and

(f) identifying a subset of candidate compounds each having an $IC_{50}$ <50$\mu$M for inhibiting a VEGF receptor while having an at least twofold $IC_{50}$ for inhibiting a control receptor.

6. The process of Claim 4 said process further comprising performing a biochemical assay wherein:

(i) lysing cells expressing said VEGF receptor;

(ii) reacting said VEGF receptor with an immobilized antibody against said VEGF receptor to isolate said receptor;

(iii) incubating said VEGF receptor with said subset of candidate compounds identified in step (a)(v) at a concentration of >50 μM;

(iv) exposing said VEGF receptor to VEGF;

(v) determining the degree of tyrosine phosphorylation of said VEGF receptor;

(vi) identifying a subset of candidate compounds which inhibit the tyrosine phosphorylation of said VEGF receptor by at least 50% compared to a control;

7. The process of Claim 4, wherein said VEGF receptor is selected from the group consisting of KDR/FLK-1 and flt-1.

8. The process of Claim 4, wherein the step of determining compounds exhibiting inhibition of said VEGF receptor comprises measuring the amount the receptor tyrosine phosphorylation of said VEGF receptor and said control receptor using an ELISA assay.

9. The process of Claim 4, wherein the $IC_{50}$ of said compound for inhibiting tyrosine phosphorylation of said VEGF receptor in step (a) is <10 μM.

10. The process of Claim 4, wherein the $IC_{50}$ of said compound for inhibiting said control receptor in step (a) is at least fivefold relative to the $IC_{50}$ of said compound for inhibiting said VEGF receptor.

11. The process of Claim 8, wherein said cultured endothelial cells are selected from the group consisting of HUV-EC and BAE cells.

12. The process of Claim 12, wherein said bioresponse is determined by measuring the $^3H$ thymidine incorporation into DNA.

13. A compound produced by the process of Claim 4.

14. The compound of Claim 13, wherein the $IC_{50}$ of said compound for inhibiting the bioresponse induced by VEGF is <10 $\mu$M.

15. The compound of Claim 13, wherein the $IC_{50}$ of said compound for inhibiting the bioresponse induced by VEGF is at least threefold higher than the $IC_{50}$ of said compound for inhibiting the bioresponse induced by a non-VEGF control ligand.

16. A pharmaceutical composition comprising a therapeutically effective amount of a compound produced by the process of Claim 4.

17. A compound comprising a therapeutically effective amount of a compound selectively inhibiting the activity of a VEGF receptor, said compound produced by a process comprising at least three increasingly selective screening steps.

18. A pharmaceutical composition comprising a therapeutically effective amount of a compound selectively inhibiting the activity of a VEGF receptor, said compound produced by a process comprising at least three increasingly selective screening steps.

19. A method for treating diseases related to unregulated or inappropriate vasculogenesis and/or angiogenesis comprising administration of an effective amount of the composition of Claim 16.

20. A method for treating diseases according to Claim 19 wherein the disease is selected from the group consisting of cancer, arthritis, diabetic retinopathy.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03378

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.2, 130; 435/4, 7.1, 7.21, 7.2, 7.92; 436/501, 536, 63; 514/1, 2, 44, 825, 866; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>A | WO 95/21613 A1 (SUGEN, INC.) 17 August 1995, especially abstract, pages 5-22 and 51-79. | 1-3, 13-20<br>----------<br>4-11 |
| X<br>---<br>A | WO 95/21868 A1 (IMCLONE SYSTEMS INCORPORATED) 17 August 1995, especially pages 25-45. | 1, 2, 13-20<br>----------<br>3-11 |
| A | WO 95/19169 A2 (SUGEN, INC.) 20 July 1995, especially pages 42-98. | 2-10 |
| X<br>--<br>A | ASANO et al. Isolation and Characterization of Neutralizing Monoclonal Antibodies to Human Vascular Endothelial Growth Factor/Vascular Permeability Factor 121 (VEGF/VPF 121). Hybridoma., 1995, Vol. 14, No. 5, pages 475-480, especially abstract and Figures 1-3 and 5. | 1-3, 13-20<br>---------<br>4-11 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>04 JUNE 1997 | Date of mailing of the international search report<br>07 JUL 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>CLAIRE M. KAUFMAN<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03378

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>A | KIM et al. The Vascular Endothelial Growth Factor Proteins: Indentification of Biologically Relevant Regions by Meutralizing monoclonal Antibodies. Growth Factors., 1992. Vol. 7, No. 1, pages 53-64, see entire document. | 1-3, 13-20<br>4-11 |
| X | GENGRINOVITCH et al. Platelet Factor-4 Inhibits the Mitogenic Activity of VEGF121 and VEGF165 Using Several Concurrent Mechanims. The Journal of Biological Chemistry, 23 June 1995, Vol. 270, No. 25, pages 15059-15065, especially abstract and page 15060. | 1 |
| X<br>--<br>A | SALEH et al. Inhibition of Growth of C6 Glioma Cells in Vivo by Expression of Antisense Vascular Endothelial Growth Factor Sequence. Cancer Research, 15 January 1996. Vol. 56, No. 2, pages 393-401, especially Figure 1 and "RESULTS" section. | 1<br>--------<br>2, 18 |
| A | JELLINEK et al. Inhibition of Receptor Binding by High-Affinity RNA Ligands to Vascular Endothelial Growth Factor. Biochemistry, 1994,.Vol. 33, No. 34, pages 10450-10456, especially abstract and pages 10455-10455. | 1, 13-20 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03378

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 12 depends on itself and is drawn to the process of claim 12, however no process is recited in the claim.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03378

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07K 2/00, 14/705, 16/28; C12N 5/06, 5/07; C12Q 1/00; A16K 31/00, 35/00, 39/395; G01N 33/15, 33/48, 22/53

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/9.2, 130; 435/4, 7.1, 7.21, 7.2, 7.92; 436/501, 536, 63; 514/1, 2, 44, 825, 866; 530/300, 350

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (MEDLINE, CAPLUS, INPADOC, PHIN)
search terms: hirth, mcmahon, shawver, kdr?, vegf?, angiogen? vascul? flt-1, flk-1, receptor?, inhibit?, suppress? tumo?, angiogen?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim 1, drawn to a process for producing a compound that inhibits angiogensis and inhibits the VEGF receptor.

Group II, claims 2-12, drawn to a process for identifying componds that inhibit angiogenesis.

Group III, claims 13-18, drawn to a compound or pharmaceutical composition produced the process of Group II.

Group IV, claims 19-20, drawn to a method of treating diseases with the pharmaceutical composition of Group III.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The process of producing a compound that inhibits angiogenesis and inhibits the VEGF receptor is not itself an advance over the prior art because GENGRINOVITCH et al.(J. Biol. Chem., 270(25):15059-15065, June 1995) describe a method of producing PF4 (platelet factor-4) by recombinant transformation of bacteria (page 15060, columns 1, 2nd full paragraph) and teach screening compounds such as PF4, two VEGF variants, and heparan, to identify compounds which inhibit the VEGF receptor (two paragraphs beginning on page 15060, column 2, with the 3rd full paragraphs). Gengrinovitch et al. show that PF4 inhibits angiogenesis and inhibits the VEGF receptor by binding heparin binding proteins such as VEGF165 (abstract). Further, the process of Group I does not share a special technical feature with the process of Group II because the processes use different steps and Group I is the production of a compound and II is the identification of a compound, but only by activity assays and not by structure. The process of Group I does not share a special technical features with the compound of Group III because the compound can be used in unrelated processes such as in purification of the VEGF receptor or in the production of an antibody to the compound. Also the compound (PF4) is not novel. The process of Group I and the method of Group IV also lack a corresponding special technical feature because they do not share process steps and are performed for different purposes. The method of Group II does not share a special technical feature with the compound of Group III because the compound is not novel and so does not provide a special technical feature. The method of Group II is further unrelated to the method of Group IV because neither process steps or purpose of using the methods are shared. The compound of Group III does not share a corresponding technical feature with the method of Group IV because the compound is not novel and because the compound may be used in many methods, including production of an antibody, purification of the VEGF receptor, or in the identification of VEGF receptor agonists.